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Recombination between chloroplast genomes of *Trachystoma ballii* and *Brassica juncea* following protoplast fusion

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Abstract We document here the presence of a recombinant plastome in a cytoplasmic male sterile (CMS) line of *Brassica juncea* developed from the somatic hybrid *Trachystoma ballii* + *B. juncea*. Restriction endonuclease digestion of the chloroplast (cp) DNA has revealed that the recombinant plastome gives rise to novel fragments in addition to the parent-specific fragments. Analysis of the 16S rRNA region by Southern hybridization shows no variation between *B. juncea, T. ballii* and the CMS line. The *rbc*L gene region of the recombinant plastome is identical to that in *T. ballii*. Analysis with probes for *psb*A and *psb*D using single and double DNA digests indicates that the hybridization patterns of the recombinant plastome are identical to those of the parents in digests obtained with some restriction enzymes, while novel bands hybridize to probes in other digests. In the *psb*A region, a *B. juncea*-specific *Pst*I site and a *T. ballii*-specific *Eco*RI site are found in the recombinant plastome. The *psb*D region of the recombinant plastome contains a *B. juncea*-specific *Hin*dIII site and *T. ballii*-specific *Bam*HI and *Hpa*II sites. These results indicate the occurrence of intergenomic recombination between the chloroplasts of *T. ballii* and *B. juncea* in the somatic hybrid from which the CMS line was developed. The recombined plastome appears to be a mosaic of fragments specific to both parents and the recombination event has occurred in the single-copy regions. These recombinational events have not caused any imbalance in the recombinant plastome in terms of chloroplast-related functions, which have remained stable over generations.

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Introduction

In sharp contrast to the extensive recombination seen between mitochondrial genomes in parasexual hybrids, intergenomic chloroplast recombination in higher plants is a rare event. Such recombination, was first reported in the progeny of an interspecific hybrid between *Chlamydomonas eugametos* and *C. moewusii* (Lemieux et al. 1981). The presence of two types of chloroplast genomes in a single plant, which differ in the relative orientation of single-copy regions, was attributed to intramolecular recombination by interconversion of the circular genome (flip-flop recombination) via inverted repeats (Palmer 1983; Mubumbila et al. 1983). Intramolecular chloroplast recombination has also been proposed to explain the occurrence of albino plants in cereals (Day and Ellis 1984). Intergenomic chloroplast recombination has been reported and analysed in detail in interspecific *Nicotiana* somatic hybrids (Medgyesey et al. 1985; Fejes et al. 1990) and intergeneric somatic hybrids of *Nicotiana* and *Solanum* (Thanh and Medgyesey 1989). Based on a preliminary Southern analysis, Kirti et al. (1993) predicted chloroplast genome recombination in a *Brassica juncea* line carrying chlorosis-corrected *B. oxyrrhina* cytoplasm. Subsequently, Kirti et al. (1995b) derived a CMS line by backcrossing the somatic hybrid *Trachystoma ballii* + *B. juncea* (Kirti et al. 1992) with male-fertile *B. juncea*, and proposed the presence of a restructured chloroplast genome in this line. In this paper, we present data on chloroplast DNA restriction fragment patterns and partial restriction maps of two gene regions (*psb*A and *psb*D), which demonstrate that intergenomic chloroplast recombination occurred after protoplast fusion between *T. ballii* and *B. juncea*, and that the CMS line thus carries a recombinant plastome.

Results

Restriction analysis

Since cpDNA restriction maps are not available for *B. juncea* or *T. ballii*, cpDNA was digested initially with various restriction enzymes to generate restriction fragment profiles. The cpDNA of the CMS line was characterized by novel, non-parental restriction fragments in addition to fragments specific and common to the parents (Table 1). Comparison of the restriction fragment profiles obtained by digestion with *Bam*HI showed that *T. ballii* has a specific 10-kb fragment which is also present in the CMS line, whereas *B. juncea* possesses specific 14.0-kb and 6.4-kb fragments. Apart from the *T. ballii*-specific fragment, the CMS line possesses two novel fragments of 3.5 kb and 1.8 kb. In the *Bgl*II digest, *B. juncea* has specific 3.5-kb and 2.1-kb fragments which are also present in the recombinant plastome. In addition to the *B. juncea*-specific fragments, the recombinant plastome has a novel 6.6-kb *Bgl*II fragment (Fig. 1). Novel fragments are also observed in the cpDNA of the CMS line on digestion with *Hpa*II, *Kpn*I and *Kpn*I + *Pst*I.

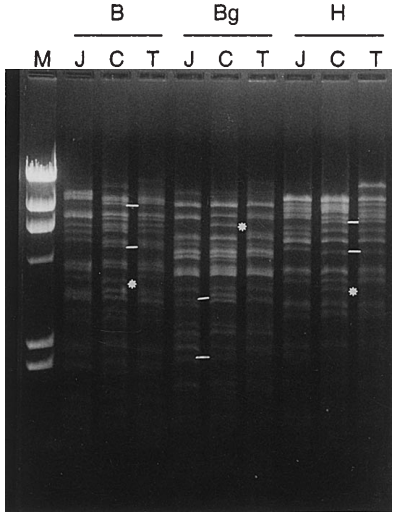

Fig. 1 Restriction patterns of chloroplast DNA from *B. juncea* (J), the CMS line (C) and *T. balii* (T). *B*, *Bam*HI: *Bg*, *Bgl*II; *H*, *Hin*dIII. CpDNA was isolated following the protocol of Kirti et al. (1995) using a high-salt buffer, digested with different restriction enzymes, run on 0.8% agarose gels on 0.5 X/TRIS-acetate buffer and stained with ethidium bromide. Corresponding fragments are connected by *horizontal lines* and novel fragments indicated by the *asterisks*

Southern analysis

Analysis of the recombinant plastome in the CMS line using cp-specific probes has also been carried out. The following probes were used in Southern analyses: *psb*A, 769-bp *Pst*I-*Xba*I fragment from spinach (Zurawski et al. 1982); *psb*D, 989-bp *Eco*RI-*Pvu*II fragment from spinach (Alt et al. 1984); *rbcL,* 1.8-kb *Eco*RI fragment from spinach (Zurawski et al. 1984); and 16S rRNA, 2.5-kb *Bgl*II fragment from pea (V. Sivareddy, unpublished data). Hybridization with the probe for the 16S rRNA gene region to single and double digests obtained with *Pst*I, *Eco*RI and *Hpa*II revealed no polymorphism in the materials used in the study. Similarly, the *rbc*L region of the CMS line is identical to that in *T. ballii* based on digestion with *Kpn*I, *Kpn*I + *Pst*I and *Hpa*II (data not shown). Southern hybridization of cpDNA and total genomic DNA fragments with *psb*A and *psb*D probes shows that the recombinant plastome of the CMS line gives a hybridization pattern similar to that of either *B. juncea* or *T. ballii* cpDNA, depending on the enzyme used. Novel hybridization patterns are also observed in some cases, indicating that the recombinant plastome has undergone changes. Based on the hybridization patterns of these two probes, restriction maps of the corresponding chloroplast genomic regions in the CMS line were constructed and compared with those of the parents.

The restriction map of the *psb*D region was constructed using *Bam*HI, *Hpa*II and *Hin*dIII. The *psb*D hybridization patterns obtained with *Hpa*II were iden-

Table 1 Parent-specific and novel restriction patterns in the plastome of the CMS line (Fragment length indicated in kb)

Restriction enzyme[a]																				
*Bam*HI			*Bgl*II			*Hin*dIII			*Hpa*II			*Kpn*I			*Pst*I			*Kpn*I + *Pst*I		
J	C	T	J	C	T	J	C	T	J	C	T	J	C	T	J	C	T	J	C	T
14	–	–																		
–	10.0	10.0	–	6.6*	–	–	–	20.0												
–	5.2	5.2				–	8.0	8.0	–	7.5	7.5							–	7.5	7.5
6.4	–	–				5.2	–	–	–	6.8*	–	–	5.6*	–				–	5.5*	–
–	3.5*	–	3.2	3.2	–	–	5.0	5.0	4.8	–	–	–	5.4	5.4						
–	1.8*	–	2.1	2.1	–	–	3.7*	–	–	2.8	2.8	4.5	–	–				–	4.0	4.0
						2.1	–	–	2.2	2.2	–				–	3.2	3.2	–	3.8	3.8
															2.5	–	–	2.6	–	–
															–	2.4*	–	–	2.5*	–
															2.3	–	–	2.4	–	–

[a] J, *B. juncea*; T, *T. balli* C, the CMS line. The minus sign indicates that the fragment is absent, the *asterisks* indicate novel fragments in the CMS genome

tical in the CMS line and *T. ballii*. The 3.0-kb *Hpa*II fragment had a *Bam*HI site in both, indicating that the *psb*D region of the recombinant plastome had originated from the wild parent. However, the pattern seen with *Hin*dIII in the CMS line was identical to that in *B. juncea*. The *Bam*HI + *Hin*dIII digest in the CMS line showed a novel fragment of 4.0 kb (Fig. 2). The various hybridization patterns are summarized in the restriction map shown in Fig. 3. This region in the CMS line possesses *Hin*dIII sites specific to *B. juncea* and *Hpa*II and *Bam*HI sites unique to *T. ballii*. Two points of exchange must be assumed to account for the novel hybridization pattern in the CMS line. One exchange occurred in the region between the *Hin*dIII site in the *B. juncea* and the *Hpa*II site in the *T. ballii* plastome. The other crossover could not be fixed in the *psb*D region of the *T. ballii* plastome with the existing data.

The restriction map of the *psb*A region is based with *Pst*I, *Eco*RI, *Xba*I and *Sma*I. The hybridization pattern given by the *psb*A probe in *Pst*I, *Pst*I + *Eco*RI, *Pst*I + *Xba*I digests of cpDNA from the CMS line was identical to that of *B. juncea*, whereas in *Eco*RI and *Eco*RI + *Xba*I digests, the hybridization pattern was similar to that of *T. ballii*. A 2.1-kb *Eco*RI fragment hybridized with the probe in the CMS line, while a 1.9-kb fragment was detected in *B. juncea* (Fig. 2). A 1.7-kb *Eco*RI-*Xba*I fragment bound to the probe in DNA from the CMS line and *T. ballii* against a 1.5 kb fragment in *B. juncea*. The same 1.4-kb *Xba*I-*Sma*I fragment was found in all the three plastomes. All these hybridization patterns are summarized in the restriction map of the *psb*A region (Fig. 4). It is evident from the map that the gene is present on a 1.0-kb *Pst*I-*Xba*I fragment in the recombinant plastome and *B. juncea*, indicating that *psb*A gene of the former has originated from *B. juncea*. The recombinant plastome also has one *T. ballii*-specific *Eco*RI site in the region around the gene, indicating that one point of exchange is located adjacent to the *Sma*I site.

Thus, restriction mapping of the *psb*A and *psb*D gene regions demonstrates that recombination has occurred between chloroplast genomes of *T. ballii* and *B. juncea* following protoplast fusion and the plastome of the CMS line represents a novel mosaic of restriction sites contributed by both the parents.

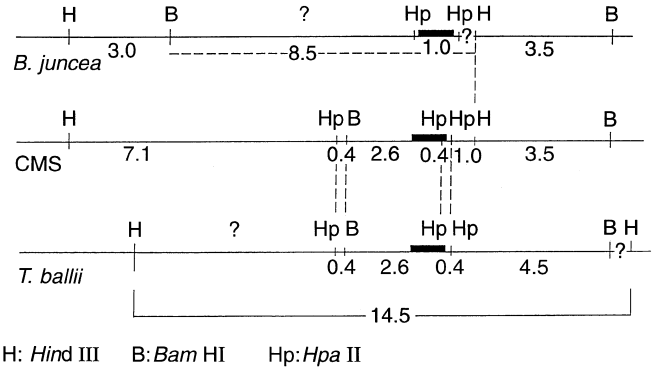

Fig. 3 Restriction map of the *psb*D region

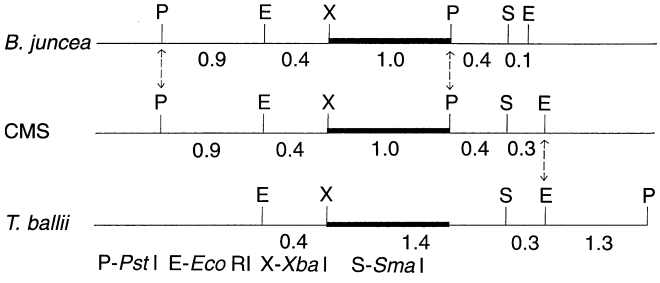

Fig. 4 Restriction map of the *psb*A region

Discussion

In flowering plants, transmission of chloroplasts is generally uniparental. In instances, where biparental inheritance was reported, attempts to recover chloroplast recombinants have failed (Kutzelnigg and Stubbe 1974; Chiu and Sears 1985). Chiu and Sears (1985) suggested that the apparent lack of recombination between cpDNAs is a reflection of a lack of plastid fusion in such cases. The lack of gene transfer between chloroplast genomes in species with biparental organelle inheritance often resulted in chlorophyll deficiencies in interspecific crosses and these were interpreted as being due to various forms of genome-plastome incompatibility (Stubbe and Herrmann 1982). Protoplast fusion

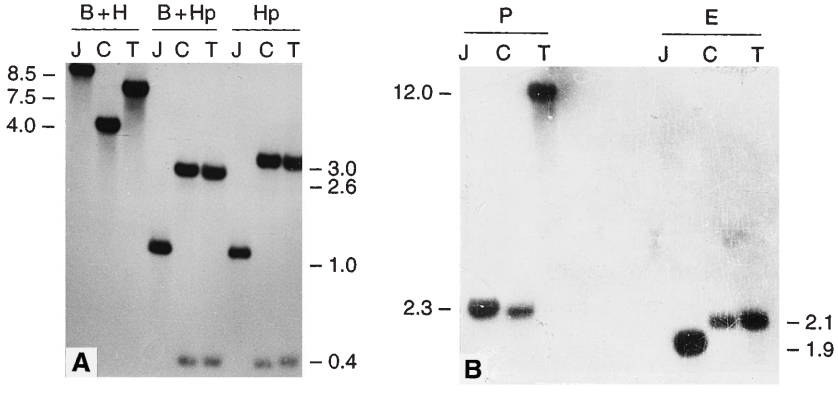

Fig. 2A, B Southern analysis of genomic DNA digests with the cp-specific probes *psb*D **A** and *psb*A **B**. Lanes are as in Fig. 1; Hp, *Hpa*II. Genomic DNA was isolated according to Saghai-Maroof et al. (1984), digested with restriction enzymes, run on 0.8% agarose gels in 0.5X TRIS-acetate buffer and blotted onto Hybond N (Amersham International) nylon membranes. Probes were labeled by nick-translation using a labeling kit from Promega

offers an opportunity for gene transfer from one species to the other by bringing together chloroplasts of divergent origin into the hybrid cell and allowing recombination to occur. Targeted introgression of alien genes into the chloroplast genome using adjacent chloroplast DNA fragments as flanking sequences in the transformation vector has been achieved through homologous recombination in tobacco (Svab et al. 1990; Svab and Maliga 1993; Staub and Maliga 1994). Recently, a native *Bt* gene has also been expressed in transplastomic plants of tobacco, together with the gene for spectinomycin resistance (Mc Bride et al. 1995). Kohler et al. (1997) have demonstrated the occurrence of tubular connections between chloroplasts. They speculated that the connections might be analogous to bacterial pili which allow the exchange of macromolecules. These tubules may have a role to play in chloroplast genome recombination.

In cases where chloroplast genome recombination has previously been reported, the event was recovered after stringent selection for recombinant plastomes. One interspecific hybrid between *N. tabacum* and *N. plumbaginifolia* pt14 was reported to possess a stable recombinant chloroplast genome (Medgyesey et al. 1985). Nucleotide sequence analysis showed that chloroplast genome recombination was extensive in this hybrid and the recombinant plastome appeared to be a mosaic of restriction sites from the two parental genomes. It did not result in DNA sequence alterations, presumably because the exchanges were homologous (Fejes et al. 1990). Thanh and Medgyesey (1989) reported limited gene transfer between chloroplast genomes in the intergeneric somatic hybrid obtained by fusing protoplasts from a light-sensitive plastome mutant (LSI) of *N. tabacum* with lethally irradiated wild-type *S. tuberosum* protoplasts. Restriction enzyme analysis of its chloroplast DNA revealed recombinant non-parental fragment patterns in addition to parent-specific fragments from both tobacco and potato. This recombinant plastome carried large and small unique regions from *N. tabacum* and the repeats from *S. tuberosum*. In contrast to these examples, which required strong selection for the recombinant plastid genomes, our example is from a fusion between untreated parental protoplasts of *T. ballii* and *B. juncea*. The present recombinant plastome also appears to be a mosaic of restriction sites in the regions of single-copy genes, as in the case of the *Nicotiana* hybrid studied by Medgyesey et al. (1985). The *psb*D gene codes for an important constituent of photosystem II (PS II) and even slight alterations (spontaneous or induced) around the 3′ UTR lead to major defects in the functioning of PS II (Gerdes et al. 1996). Similarly, *psb*A encodes the D1 protein that binds to the PS II reaction centre. These are essential functions and any change in the gene sequence may not be tolerated. Since the CMS line carrying the recombinant plastome did not suffer from any abnormalities, such as chlorosis, reduced vigour etc., it is likely that the plastome carried a delicately balanced set of genes crucial for proper function from both the parents, which essentially complement each other. The recombinant plastome appears to be very stable, since similar cpDNA restriction and Southern hybridization patterns have been obtained over several seasons. The occurrence of a similar reorganization of chloroplast genes, which preserves the functionality of the plastome, has also been assumed in the case of the 'potacco' plastome (Thanh and Medgyesey 1989).

Further work on cloning and sequencing novel chloroplast DNA fragments should give further insight into the phenomenon of chloroplast genome recombination between *B. juncea* and *T. ballii*.

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